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REMARKS

Claims 1, 7, 9, 11-15 and 19-24 stand rejected under 35 USC 112, first paragraph, as failing to comply with the written description requirement. Specifically, the Examiner states that the addition of the phrase "residues +1 to -368 of SEQ ID NO: 3" constitutes new matter because SEQ ID NO: 3 comprises nucleotides 1 to 2474. The nomenclature used in the claims employs a more scientific numbering method than that used to define the numbering in SEQ ID NO: 3. Specifically, the "+/-" indicates "from the start of transcription". It is a straightforward procedure to determine the nucleotide sequence of the promoter fragment relative to SEQ ID NO: 3 from the description of the deletion fragments in Example 5 on pages 37 and 38 of the specification or from the information set out in Figure 12.

The sign "+1" in "+1 to -368" indicates the start of transcription. The ATG indicated on Figure 12 (at the 5' end of the cartoon representing the genomic structure) represents the start of translation. The line at the foot of Figure 12 indicates that the ATG ends at nucleotide +90. The ATG at the end of SEQ ID NO: 3 is at nucleotides 2471 to 2473. Accordingly, nucleotides 2471 to 2473 of SEQ ID NO: 3 are nucleotides 88 to 90 of Figure 12. Thus, the sign "+1" of Figure 12 referred to in the claims is nucleotide 2384 of SEQ ID NO: 3. Subtracting a further 368 nucleotides indicates that nucleotide 2016 of SEQ ID NO: 3 corresponds to -368 of Figure 12and reference to "-368" in the claims. In order to advance prosecution and to be consistent with the specification, claims 1, 7 and 15 have been amended to replace "residues of +1 to -368 of SEQ ID NO: 3" with "residues 2016 to 2384 of SEQ ID NO: 3". Accordingly, this rejection should be withdrawn.

Claims 1, 7, 9, 11-15 and 19-24 stand rejected under 35 USC 112, first paragraph, as being non enabled by the specification. Specifically, the Examiner states that the specification allegedly fails to provide enablement for claimed sequences other than SEQ ID NO: 3. In particular, the Examiner alleges that the identification of molecules that meet the claim limitations would be a highly unpredictable endeavor. The Examiner has cited a number of documents that allegedly

support her argument in relation to the unpredictability of the art. However, none of these documents relate either to promoter sequences or to plant promoter sequences. Applicants note that with respect to promoter sequences it is widely known in the art that promoter regions are very flexible. This means that point mutations do not normally alter the expression intensity or pattern of expression of the promoter. This is because promoters have modular structures joined by linking DNA regions that have little or no function other than to serve as "spacers" among the functional modules. It is for this reason that it would be reasonably predictable how to design a promoter with some sequence variation yet maintaining the strength of the pGEL1 promoter. This is illustrated in the specification which describes a comprehensive family of deletion mutants that still confer high expression levels of ACC synthase.

In addition, enclosed is a copy of *Cazzonelli et al.*, Transgenic Research, 14:941-967, 2005 providing information from the inventors regarding the subject promoter. The Examiner's attention is particularly directed to page 946, column 2, second full paragraph which illustrates how regulatory elements within the promoter sequences can be identified.

Finally, in order to advance prosecution, claims 1, 7 and 15 have been amended to specify that that the promoter "confers, activates or enhances expressions of a structural gene or other nucleic acids in a plant cell." This further defines claimed sequences through there functionality.

Since the claims as amended are enabled by the specification as filed, this rejection of claim 1, 7, 9, 11-15 and 19-24, should be withdrawn.

In view of the above, each of the presently pending claims in this application is believed to be in immediate condition for allowance. Accordingly, the Examiner is respectfully requested to withdraw the outstanding rejection of the claims and to pass this application to issue. If it is determined that a telephone conference would expedite the prosecution of this application, the Examiner is invited to telephone the undersigned at the number given below.

In the event the U.S. Patent and Trademark Office determines that an extension and/or other relief is required, applicants petition for any required relief including extensions of time and authorizes the Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to **Deposit Account No. 03-1952** referencing docket no. **229752001300**.

Dated: December 6, 2006

Respectfully submitted,

By_____

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Characterization of a strong, constitutive mung bean (*Vigna radiata* L.) promoter with a complex mode of regulation *in planta*

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Abstract

We report the cloning and characterization in tobacco and *Arabidopsis* of a *Vigna radiata* L. (mung bean) promoter that controls the expression of *VR-ACS1*, an auxin-inducible ACC synthase gene. The *VR-ACS1* promoter exhibits a very unusual behavior when studied in plants different from its original host, mung bean. GUS and luciferase *in situ* assays of transgenic plants containing *VR-ACS1* promoter fusions show strong constitutive reporter gene expression throughout tobacco and *Arabidopsis* development. *In vitro* quantitative analyses show that transgenic plants harboring *VR-ACS1* promoter–reporter constructs have on average 4–6 fold higher protein and activity levels of both reporter genes than plants transformed with comparable CaMV 35S promoter fusions. Similar transcript levels are present in *VR-ACS1* and CaMV 35S promoter lines, suggesting that the high levels of gene product observed for the *VR-ACS1* promoter are the combined result of transcriptional and translational activation. All tested deletion constructs retaining the core promoter region can drive strong constitutive promoter activity in transgenic plants. This is in contrast to mung bean, where expression of the native *VR-ACS1* gene is almost undetectable in plants grown under normal conditions, but is rapidly and highly induced by a variety of stimuli. The constitutive behavior of the *VR-ACS1* promoter in heterologous hosts is surprising, suggesting that the control mechanisms active in mung bean are impaired in tobacco and *Arabidopsis*. The 'aberrant' behavior of the *VR-ACS1* promoter is further emphasized by its failure to respond to auxin and cycloheximide in heterologous hosts. *VR-ACS1* promoter regulatory mechanisms seem to be different from all previously characterized auxin-inducible promoters.

Abbreviations: ABA – abscisic acid; ACC1 – aminocyclopropane-1-carboxylate; CaMV – Cauliflower Mosaic Virus; CHX – cycloheximide; GUS-β – glucuronidase; LUC – luciferase; 4-MU4 – methylumbelliferone; PCR – polymerase chain reaction; UTR – untranslated region; X-gluc – 5-bromo-4-chloro-3-indolyl-β-*D*-*glucuronide*

Introduction

Even though important advances have been made in understanding the molecular mechanisms controlling plant gene expression, there is much yet to be learned. Very few promoters are well characterized compared to the number of genes that have been studied. This is in part due to the extremely complex nature of the interactions that

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take place between the large number of *cis*-elements that contribute to promoter function and the multitude of nuclear transcription factors that associate with them. Sometimes, as in the case of some auxin-induced genes, several *trans*-factors can interact to exert an effect through a specific *cis*-element, resulting in either repression, induction or super-induction of the gene (Rogg & Bartel, 2001; Tiwari et al., 2003, 2004). Knowledge of the mechanisms of gene regulation is crucial to further our understanding of plant growth and development and is especially critical for the development of plant biotechnology. Precise regulation of transgenes in genetically modified plants is necessary in order to produce crops with enhanced traits such as improved yield, disease resistance and stress tolerance. Both constitutive and tissue specific promoters are needed, depending on the biotechnological application being developed.

Constitutive promoters identified from plant viruses, such as the Cauliflower Mosaic Virus (Odell et al., 1985; Kay et al., 1987; Benfey & Chua, 1990; Holtorf et al., 1995; Wilmink et al., 1995), Cassava Vein Mosaic Virus (Verdaguer et al., 1998), Figwort Mosaic Virus (Sanger et al., 1990; Maiti et al., 1997; Bhattacharyya et al., 2002), Peanut Chlorotic Streak Virus (Maiti & Shepherd, 1998; Bhattacharyya et al., 2003), Taro Bacilliform Virus (Yang et al., 2003), Cestrum Yellow Leaf Curling Virus (Stavolone et al., 2003), Mirabilis Mosaic Virus (Dey & Maiti, 1999), Sugarcane Bacilliform Badnavirus (Tzafrir et al., 1998; Schenk et al., 1999, 2001) and Subterranean Clover Stunt Virus (Schunmann et al., 2003) are widely used in biotechnology. This is in part due to a scarcity of well characterized strong constitutive plant promoters. More importantly, this may reflect the relative simplicity of viral replication mechanisms in comparison to the complexity of plant regulatory systems required during development and in order to survive a multitude of abiotic and biotic challenges. Several constitutive promoters have been cloned and characterized in plants, including promoters from the translation initiation factor 4A gene of tobacco (Mandel et al., 1995), rice *OsPSK* gene (Yang et al., 2000), actin genes from rice (Zhang et al., 1991) and *Arabidopsis* (An et al., 1996), histone H2B gene (Rasco-Gaunt et al., 2003), ubiquitin genes in *Arabidopsis* (Callis et al., 1990) and maize (Christensen et al., 1992), GOS2 gene of rice (de Pater et al., 1992) and the cryptic tCUP promoter from tobacco (Foster et al., 1999, 2003; Wu et al., 2001, 2003). The tCUP promoter is one of the strongest constitutive plant promoters that has been thoroughly studied and its regulation is quite different to that of other constitutive plant promoters. New and well characterized plant promoters will be especially useful when 'gene stacking' is being pursued or licensing and intellectual property issues need to be avoided.

We have previously cloned a cDNA, *VR-ACS1*, encoding an ACC synthase protein from auxin-treated mung bean hypocotyls (Botella et al., 1992). Even though very little *VR-ACS1* transcript is detectable under normal physiological conditions, expression is strongly induced by mechanical stress (touch), auxins, cycloheximide (Botella et al., 1992, 1995), kinetin (Yi et al., 1999), ABA, wounding, inhibitors of ethylene biosynthesis (Kim et al., 1997a, b) and salt and desiccation stress (Cazzonelli, unpublished). Nuclear run-on assays show that the observed induction is due to transcriptional activation rather than post-transcriptional events (Yi et al., 1999). It is also known that ethylene (Yoon et al., 1997) and brassinosteroids (Yi et al., 1999) can suppress induction of *VR-ACS1* gene expression in response to some of the above stimuli. Protein kinase and phosphatase inhibitors can relieve the ethylene induced suppression, which suggests that phosphorylation is important in regulation of *VR-ACS1* (Kim et al., 1997a). All available data shows that the *VR-ACS1* gene displays highly complex regulation *in planta*, making it a very interesting candidate for the study of promoter function and transcription regulatory mechanisms. Several plant ACC synthase promoters have been isolated and characterized and they have all been shown to be tissue-specific, inducible or developmentally regulated (Lincoln et al., 1993; Rodrigues-Pousada et al., 1993; Gil et al., 1994; Abel et al., 1995; Yi et al., 1999; Yoon et al., 1999; Tsuchisaka & Theologis, 2004).

We report here the isolation and characterization of the mung bean *VR-ACS1* promoter. We show that a 2.5 kb fragment of the promoter drives high constitutive expression of β-glucuronidase (GUS) in tobacco and *Arabidopsis*, as well as luciferase in tobacco. On average, plants harboring the reporter genes under the control of the *VR-ACS1* promoter showed 4–6 times higher

activity levels than plants containing CaMV 35S driven reporter genes. The high levels of activity observed in *VR-ACS1* promoter transgenic plants are the result of strong transcriptional activity combined with a translational enhancer-like activity from the *VR-ACS1* 5′-untranslated leader sequence.

Materials and methods

Isolation and cloning of the VR-ACS1 promoter

Genomic DNA was isolated from young mung bean (*Vigna radiata* L.) leaf tissue using the nucleic acid extraction method described by Cazzonelli et al. (1998), followed by further purification using CsCl isopycnic centrifugation. Genomic DNA (20 μg) was digested to completion with *Hind*III and recircularized using 9 Weiss units of Promega's T4 DNA ligase, in 400 μL of ligation buffer, under conditions that favor the formation of monomeric circles (Collins & Weissman, 1984). An initial PCR amplification was performed in a reaction mixture containing 60 mM Tris–SO_4 pH 9.1, 2 mM $MgSO_4$, 18 mM $(NH_4)_2SO_4$, 0.2 μM of each dNTP, 0.2 μM of primers NSE1 (5′-GCGGATCCATCTTGGACAACAAGGGAGT T-3′) and NSE2 (5′-TAGGATCCAGAAAGA CACTGAGAACCGTGG-3′), 1.75 μl of 'Elongase enzyme mix' (Life Technologies) and approximately 360 ng of recircularized DNA in a total volume of 50 μl. 45 amplification cycles consisting of 30 s at 94°C and 480 s at 68°C were performed. 1 μL of the products of this PCR reaction were further reamplified using a set of nested primers NSE3 (5′-ACGGATCCGGTGTATGTGGTTA-GAGTGTG-3′) and NSE4 (5′-CAGGATCCAGA CATAGAGTGTGACCGCAA-3′), in a PCR consisting of 35 cycles of 30 s at 94°C, 30 s at 62°C and 480 s at 68°C.

The PCR reamplification reaction was fractionated by electrophoresis and a product approximately 4 kb in size purified. A fragment assembly map of the 4 kb product was constructed. The purified 4 kb product was digested with *Spe*I and blunt ended before cloning the digestion products into pGEM11 (cut with *Xho*I and blunt ended), resulting in the creation of two fragments approximately 1.1 kb (p1.1GEM11) and 1.4 kb (p1.4GEM11). The *Spe*I 1.4 kb clone from p1.4GEM11 was excised with *Spe*I and *Hind*III (blunt ended) and cloned into p1.1GEM11 containing the *Spe*I 1.1 kb fragment linearized with *Spe*I and *Sal*I (blunt ended). As a result, the full length promoter region (−2383 bp) plus the 5′ untranslated leader sequence from *VR-ACS1* gene (+85 bp) was reconstructed (Genbank accession #AY819645).

Construction of promoter–reporter gene fusions

To facilitate the cloning process into binary vectors, intermediate vectors were designed. A promoterless-GUS reporter gene cassette was prepared by excising the *gusA* gene and Nos terminator (NosT) from pBI121 with *Sma*I/*Eco*RI and the fragment ligated into pBS cut with *Sma*I/*Eco*RI, producing the intermediate vector pGuNt. A promoterless-LUC reporter gene fusion was prepared by excising the NosT sequence from pGuNt with *Eco*RI/*Ecl*136II and cloning it into pBS digested with *Eco*RI and *Pst*I (blunt ended) producing the vector pBS:NosT. The *luc* gene was excised from the pSP-*luc*+NF fusion vector (Promega) using *Xba*I/*Nco*I, cohesive ends removed and ligated into pBS:NosT linearized with *Sma*I. This intermediate vector was named pLuNt.

The binary vector pPZP111 was selected as the transformation vehicle for promoter–reporter gene fusions (Hajdukiewicz et al., 1994). **pPZP2.5GuNt.** An intermediate vector p2.5GuNt was prepared by cloning the 2.5 kb *VR-ACS1* promoter (excised from p2.5GEM11 with *Bam*HI) into pGuNt. The promoter-GUS-NosT fragment was excised from p2.5GuNt by digesting with *Sac*II, the 3′ overhang removed and digested with *Hind*III. The resulting fragment was cloned into pPZP111 cut with *Ecl*136II/*Hind*III producing the binary vector pPZP2.5GuNt. **pPZP2.5LuNt.** An intermediate vector p2.5LuNt was prepared by cloning the 2.5 kb *VR-ACS1* promoter (excised from p2.5GEM11 with *Bam*HI) into pLuNt. The binary vector pPZP2.5LuNt was constructed by excising the 2.5 kb *VR-ACS1* promoter-Luc-NosT fragment from p2.5LuNt with *Sac*I/*Hind*III and ligating it into pPZP111. All intermediate and final constructs were sequenced.

Control binary vectors containing a CaMV 35S-reporter gene fusion were also constructed in the pPZP111 binary vector. **pPZP35SGuNt.** pPZP35SGuNt was created by removing the

35S-GUS-NosT from pBI121 with *Hind*III/*Eco*RI and cloning into pPZP111. **pPZP35SLuNt.** For construction of pPZP35SLuNt, the CaMV 35S promoter was excised from pPZP35SGuNt with *Hind*III, the linearized product blunt ended and subsequently digested with *Bam*HI. The resulting fragment was cloned into pLuNt digested with *Xba*I (blunt ended) and *Bam*HI. This intermediate vector was named p35SLuNt. Finally, p35SLuNt was digested with *Sac*I/*Hind*III and the resulting CaMV35S-LUC-NosT fragment ligated into pPZP111.

A series of six 5′ deletions of the *VR-ACS1* promoter were fused to the *gusA* reporter gene in the pPZP111 binary vector. **pPZP1.1GuNt (–1032).** The p1.1GuNt intermediate vector was constructed by digesting p1.1GEM11 with *Spe*I/*Bam*HI and ligating a –1032 base pair promoter fragment (plus the 5′UTR) directly into pGuNt (*Xba*I/*Bam*HI). p1.1GuNt was subsequently digested with *Not*I, blunt ended and the promoter-GUS-NosT fragment excised with *Hind*III and subsequently cloned into pPZP111 (*Ecl*136II/*Hind*III). **pPZP0.88GuNt (–791).** A promoter fragment containing 791 bp from the start of transcription (plus the 5′UTR) was amplified by PCR using the primers NSE3 and DEL#3 (5′-GCGAGCTCATCATTCAATACGAGTAATTC-3′) and a proof reading enzyme. The fragment was cloned into the *Eco*RV site of pBS II. The promoter deletion was then excised from pBS II with *Ecl*136II and *Bam*HI and cloned into pGuNt digested with *Spe*I (blunt ended), and *Bam*HI. The resulting intermediate vector (p0.88GuNt) was subsequently digested with *Xba*I/*Hind*III and the promoter-GUS-NosT fragment cloned into pPZP111. **pPZP0.7GuNt (–611).** Primers NSE 3 and DEL#2 (5′-AGCTCTAGAGTAACGGGCTTAGACTGATG -3′) were used to amplify 611 base pairs of the *VR-ACS1* promoter (plus the 5′UTR). The PCR fragment was digested with *Bam*HI/*Xba*I and ligated into pGuNt producing the intermediate vector p0.7GuNt. p0.7GuNt was digested with *Xba*I/*Hind*III and the promoter-GUS-NosT fragment cloned into pPZP111. **pPZP0.45GuNt (-366).** The intermediate vector p0.45GuNt was produced by digesting p1.1 GEM11 with *Acc*I, removing cohesive ends, digesting with *Bam*HI and the purified –366 bp *VR-ACS1* promoter deletion (plus the 5′UTR) cloned into pGuNt digested with *Bam*HI and *Xba*I (blunt ended). The binary vector pPZP0.45GuNt was made by excising the promoter-GUS-NosT fusion from p0.45GuNt, with *Bam*HI and a restored *Xba*I and cloning into pPZP111. **pPZP0.23GuNt (–141).** p1.1GEM11 was digested with *Cla*I (blunt ended) and *Bam*HI. The excised fragment (containing –141 bp upstream of the start of transcription plus the 5′UTR) was cloned into pGuNt digested with *Bam*HI and *Spe*I (blunt ended) to produce p0.23GuNt. The promoter-GUS-NosT fragment was excised with *Bam*HI/*Xba*I and cloned into pPZP111.

pVRACS1-iGUS. Contains the full length *VR-ACS1* promoter including its 85 bp 5′UTR fused to the intron-containing *gusA* reporter gene (Tanaka et al., 1990) and nopaline synthase terminator. **p35SPAE–iGUS.** Contains the CaMV 35S promoter fused to a pectinacetylesterase gene (PAE) 5′UTR from *Vigna radiata* (Breton et al., 1996) fused to iGUS. Vector sequences and detailed maps are available from the authors upon request.

Transformation of tobacco and Arabidopsis *and selection of lines for analysis*

Nicotiana tabacum cv. Wisconsin 38, Ti68 was transformed using leaf discs as previously described (Svab et al., 1995; Horsch et al., 1988). Kanamycin was used for selection of transformed tissues. After regeneration under selection, all tobacco T_0 primary transformants were analyzed by histochemical GUS or chemiluminescent luciferase assays. A young leaf was excised from each transformant and the intensity/pattern was visually scored from 0 to 3. A table was constructed defining the intensity and patterns of reporter gene activity for each line, from which a consensus was determined. Representative lines belonging to this majority consensus were selected and homozygous individuals obtained for further analysis. This procedure was followed for all constructs studied in our work, including *VR-ACS1*, CaMV 35S and promoter deletion constructs. The number of gene insertions was determined by segregation ratio analysis.

Transformation of *Arabidopsis thaliana* ecotype Columbia was performed by vacuum infiltration (Bechtold et al., 1993). Plants were grown under long day conditions (16 h photoperiod, 21°C). Transgenic lines were selected by kanamycin resistance. Seedlings surviving the selection were

considered independent transgenic lines. All resistant plants were grown and the first cauline leaf histochemically assayed for GUS activity. A consensus was determined for each construct and representative lines selected to obtain homozygous individuals.

β-Glucuronidase (GUS) assays

Histochemical assays were performed according to Jefferson (1987), with minor modifications. Tissues from young plants were vacuum infiltrated for 15–20 min in a reaction mixture containing 50 mM Na_2HPO_4 pH 7.0, 0.5% Triton X-100, 10 mM EDTA sodium salt, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6.3H_2O$, and 2 mM X-Gluc. Soil grown plants were pretreated in cold 90% acetone for 15–30 min (Sieburth & Meyerowitz, 1997). After acetone pretreatment, the tissues were washed for 5 min in 50 mM Na_2HPO_4 (pH 7.5) rinse buffer containing 0.5 mM $K_3Fe(CN)_6$ and 0.5 mM $K_4Fe(CN)_6 \cdot 3H_2O$ before incubating in the GUS assay mixture described above. Infiltrated tissues were incubated in the GUS assay mixture for 24 h at 37°C or until the blue indigo dye precipitate was observed. Tissues were immediately cleared by washing in a series of ethanol steps and stored in 60% ethanol.

GUS fluorometric assays were carried out according to the method described by Jefferson et al., (1986) using microtitre well plates, 1–2.5 μg of protein, and 1 mM 4-methyl-umbelliferyl glucuronide as a substrate. GUS activity was determined by continuous assays using Labsystems Fluoroscan *Ascent* FL microfluorometer reader and software. Successive fluorescence readings were determined at a wavelength of 365/455 nm over a 70 min period. For all the results shown in this work, samples were extracted twice and each extraction was assayed in duplicate.

Luciferase (LUC) assays

In vivo LUC assays were conducted according to Mudge and Birch (1998). For assays on plants, camera exposures were preceded by a 200 s dark incubation to reduce light emission from green tissues due to chlorophyll fluorescence and followed by a 500 s exposure to detect luciferase expression. Luminescent specimens were imaged in a dark chamber using a laser-thinned, back illuminated, liquid nitrogen cooled, slow scan CCD chip camera and associated image processing computer.

In vitro LUC assays were conducted according to Luehrsen and Walbot (1993). Plant material (50–100 mg) was collected and frozen immediately in liquid nitrogen. Whilst frozen, the plant material was ground to a fine powder and homogenized in 300 μl of 300 mM potassium phosphate buffer pH 7.8, 1 mM EDTA, 7 mM DTT, 0.3% triton-X100 and 10% glycerol. The homogenate was incubated for 1 h to allow complete extraction of the luciferase protein, and the supernatant assayed immediately. The assay reaction was prepared in microtitre plates containing 10 μl of supernatant and 90 μl of a master mix (20 mM tricine, 5 mM $MgCl_2$, 0.1 mM EDTA, 3.3 mM DTT, 0.27 mM CoEnzyme A, 0.5 mM ATP and 0.5 mM luciferin). A delay time of 2 s was allowed before the relative amount of light units (RLU) emitted was determined over a 10 s interval. The integrated sum of RLU was expressed as a function of gram fresh weight or total soluble protein. For all the results shown in this work, samples were extracted twice and each extraction was assayed in duplicate.

Preparation of tobacco cell cultures and particle bombardment

Tobacco cells were grown in 100 ml NT1 media (4.3 g/l MS salts (Murashige & Skoog, 1962), 30 g/l sucrose, 1 ml/l BAP [1mg/ml stock], 200 μl/l 2.4D [1mg/ml stock], Millers stock 3 ml/l [60 g/l KH_2PO_4], Inositol stock 10 ml/l [10 g/l Myo-inositol. 0,1 g/l Thiamine] pH 5.5–5.7 [KOH]) for 5 days at 28°C. Tobacco cell cultures (3 ml) were vacuum filtrated onto filter paper and incubated on OS media (2.17 g/l MS salts, 500 μl MS vitamins [1000× stock], 36.4 g/l mannitol, 5g/l phytagel, pH 6.5–7.0) 4 h prior to bombardment. Tobacco cells were transferred to RM solid media 2 h after bombardment (2.17 g/l MS salts, 1 ml MS vitamins [1000× stock], 30g/l sucrose, 5 g/l phytagel, pH 6.5–7.0) and 300 μl of RM broth (2.17 g/l MS salts, 1 ml MS vitamins [1000× stock], 30g/l sucrose, pH 6.5–7.0) was layered on top of the tobacco cells. Tungsten particles (20 mg) were washed in ethanol, rinsed and resuspended in 200 μl of water. 50 μl of tungsten

suspension was mixed with 10 µl (0.8 µg/µl) of test DNA and 10 µl (0.8 µg/µl) of normalizing DNA. The suspension was vortexed before adding 50 µl of $CaCl_2$ and 20 µl of 0.1 M spermidine. After allowing the tungsten particles to settle, the supernatant was removed and 6 µl of the suspension was placed on a filter for bombardment. A particle inflow gun was used for bombardment driven by helium gas. A pressure of 1400 kPA was used for bombardment of tissues at a distance of 12.5 cm between the outlet of the gun and the explant. A baffle was placed above the tobacco cell disc and the vacuum in the chamber brought down to −29 kPA immediately before shooting. Two replicate bombardments were performed for each construct and for each bombardment activities were measured in triplicate.

Northern and Southern analysis

Northern and Southern analyses were performed as described by Sambrook et al. (1989). For the induction assays, leaf disks were cut out of transgenic tobacco plants from line 2.5G#7-3 containing the *gusA* reporter gene under the control of the full length *VR-ACS1* promoter. Leaf disks were either immediately frozen (control 0 h) or floated in a solution containing water (control 3 h), 250 µM IAA, 100 mM NaCl, or 50 µM cycloheximide. After 3 h of treatment, tissues were frozen until RNA extractions and GUS fluorometric assays were performed as described above.

Results

Isolation and identification of the VR-ACS1 promoter

Southern analysis of *Hind*III digested mung bean genomic DNA probed with a fragment of the *VR-ACS1* cDNA, revealed two signals approximately 5.2 kb and 4.3 kb in size of which the larger fragment corresponded to the 5′ end of the *VR-ACS1* cDNA. Mung bean genomic DNA was therefore digested with *Hind*III, circularized and a 4 kb DNA fragment amplified using the inverse-PCR technique and two different sets of nested primers (NSE1/NSE2 and NSE3/NSE4). Attempts to clone the 4-kb fragment were unsuccessful, therefore the amplicon was digested with either *Xba*I or *Spe*I and the resulting restriction fragments cloned into plasmid vectors. Sequence analysis of the different *Xba*I and *Spe*I clones allowed determination of the complete sequence of the promoter and reconstruction of a fragment expanding 2.5 kb upstream of the AUG start codon of the *VR-ACS1* gene (Figure 1a). It is important to note that the 3′ end of the reconstructed promoter region contained the 5′-untranslated region (5′UTR) of the *VR-ACS1* cDNA (Botella et al., 1992). The 2468 bp promoter fragment is referred hereafter as 'full length' to differentiate it from the deletion constructs described later.

The identity and integrity of the *VR-ACS1* promoter was confirmed by Southern analysis. Mung bean genomic DNA was digested with restriction endonucleases *Cla*I, *Bam*HI, *Hind*III, or *Bcl*I and successively probed with a fragment of the *VR-ACS1* promoter (Figure 1b, probe AX) and a partial *VR-ACS1* cDNA fragment (Figure 1b, probe AE). The patterns of the hybridization signals observed in each lane agree with the deduced restriction map further confirming the integrity of the promoter.

Analysis of the *VR-ACS1* promoter sequence identified a putative start of transcription site based on eukaryotic consensus sequences (Bucher & Trifonov, 1986). In addition, the putative start of transcription site correlates well with cDNA sequence data obtained from a cDNA library screen which identified several clones of *VR-ACS1* starting at the same nucleotide (Botella et al., 1992). Though lacking direct experimental confirmation, this putative transcription start site was chosen to determine the position +1 in the sequence (Figure 2). There are putative TATA and CAAT boxes at positions −34 and −96, respectively, which is in good accordance with other published core promoter consensus sequences (Grierson & Covey, 1988; Yamaguchi et al., 1998). An 85 bp leader sequence with 62% A–T content separates the transcription and translation start sites. Putative repressor-, activator- and enhancer-like regulatory elements were identified within the *VR-ACS1* promoter core regulatory domain such as the YY1 *cis*-element (CCA/TTNTTNNNA/T) that binds the Yin Yang-1 (YY1) transcription factor (Shrivastava & Calame, 1994). In maize, a YY1-like protein

acts as a suppressor of *rbcS-m3* expression in mesophyll cells (Xu et al., 2001) and a putative ortholog has been found in *Arabidopsis* (Englbrecht et al., 2004).

Besides the elements found within the core promoter, *in silico* database searches (Schug & Overton, 1997; Higo et al., 1999; Rombauts et al., 1999) revealed the *VR-ACS1* promoter sequence contains a number of regulatory motifs that have been previously identified in other plant promoters (Guilfoyle, 1997). Figure 2 displays a number of common putative promoter *cis*-acting elements, which can be broadly classified into two groups; (1) auxin-responsive like elements, and (2) ABA, dehydration and temperature-responsive like elements. Auxin-responsive like sequences identified in the *VR-ACS1* promoter region include four AuxRE like motifs (Liu et al., 1994) and an NDE motif (McClure et al., 1989) (Figure 2). Among the second group of *cis*-acting elements are two G-box-like elements (Menkens et al., 1995), a seed-specific Sph/RY-like motif (Baümlein et al., 1992), MYB (Iwasaki et al., 1995) and MYC (Murre et al., 1989) recognition sites, and low temperature stress (LTRE) (Jiang et al., 1996) and dehydration stress (DRE) responsive elements (Yamaguchi-Shinozaki & Shinozaki, 1994). In addition to the *cis*-elements mentioned above, the *VR-ACS1* promoter sequence contains two large inverted repeat sequences (38–48 nt and 16 nt each) with greater than 75% homology, as well as three direct repeat structures (7, 9 and 10 nt each). The position of these regulatory elements in the promoter is shown in Figure 2.

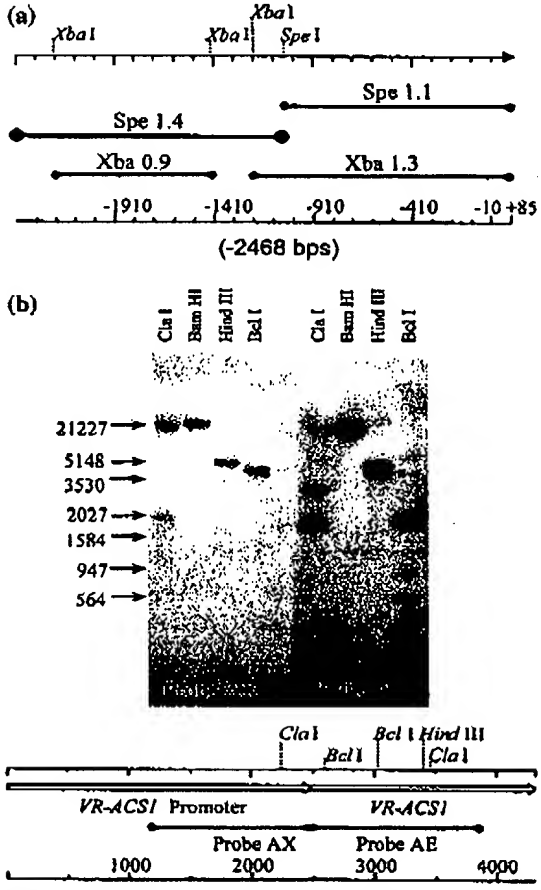

Figure 1. Fragment assembly map and Southern analysis of the *VR-ACS1* promoter. (a) Two *Spe*I fragments of the 4 kb PCR product were used to reconstruct 2468 bp of the *VR-ACS1* promoter. (b) Southern analysis of the *VR-ACS1* promoter. Mung bean genomic DNA was digested with restriction endonucleases *Cla*I, *Hind*III, *Bam*HI or *Bcl*I, separated on a 0.8% agarose gel and transferred by capillary action to a hybond-N membrane. The blot was sequentially probed with ^{32}P-labelled fragments of the *VR-ACS1* promoter (probe AX) and *VR-ACS1* cDNA (probe AE).

Developmental characterization of the VR-ACS1 promoter in tobacco

A transcriptional fusion of the full length *VR-ACS1* promoter (including the 5′-untranslated region) was made with either the β-glucuronidase (*gusA*) (Jefferson et al., 1986) or firefly luciferase (*luc*) (Ow et al., 1986) reporter genes in the pPZP111 binary vector (Hajdukiewicz et al., 1994) and a number of independent transgenic tobacco lines produced. Our strategy to produce and choose representative transgenic lines was carefully planned to avoid any artifacts. For each of the binary constructs, leaf tissue from a large number of independent T_0 transformants (32 for the *VR-ACS1*:GUS construct and 37 for the *VR-ACS1*:LUC construct) were assayed for reporter gene activity (data not shown). The Cauliflower Mosaic Virus (CaMV) 35S promoter was also fused to either the *gusA* or *luc* genes as a positive control. Again, leaves from a large number of independent primary transformants (39 for the CaMV35S:GUS construct and 35 for the CaMV35S:LUC construct) were analyzed.

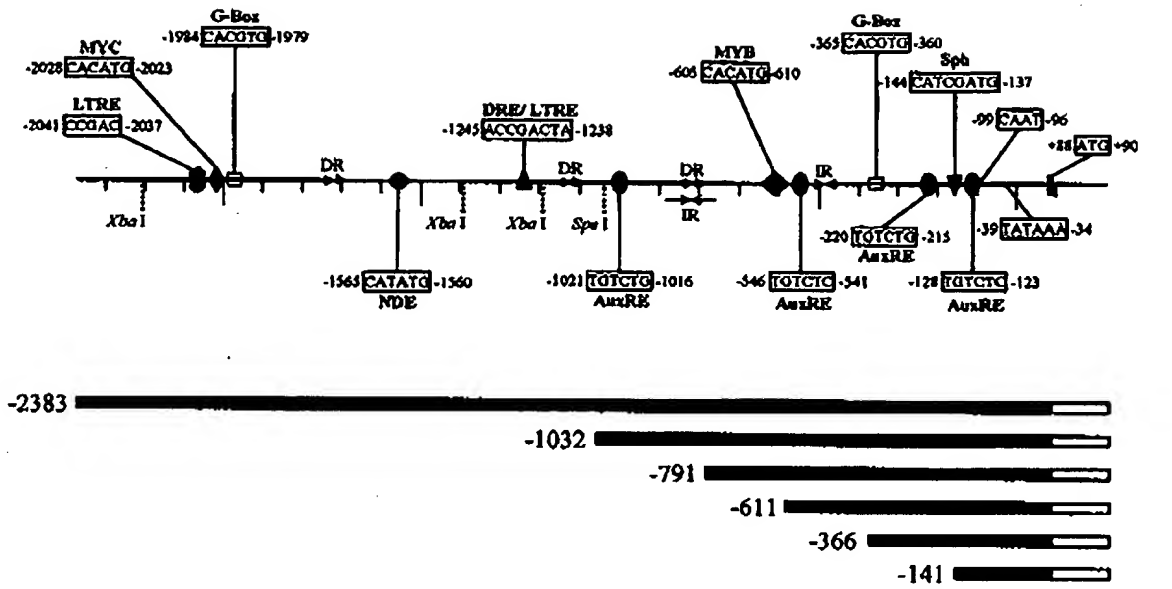

Figure 2. Map of putative *VR-ACS1 cis*-regulatory elements and deletion derivatives. Translation start ATG, CAAT and TATA motifs are boxed in bold. Auxin-like regulatory elements identified in the *VR-ACS1* promoter include four AuxRE (TGTCTC) elements (Liu et al., 1994) and one NDE (CATATG) (McClure et al., 1989) element. Recognition sequences for ABA, temperature and dehydration-like elements include the G-box/ABRE (CACGTG) (Hattori et al., 1992; Menkens et al., 1995), DRE –drought responsive element (TACCGACAT) (Yamaguchi-Shinozaki & Shinozaki, 1994), LTRE –low temperature responsive element (CCGAC) (Jiang et al., 1996) and Myb (YAACT/GG) and Myc (CANNTG) binding sites (Iwasaki et al., 1995). Inverted repeat (IR) and direct repeats (DR) are shown.

Visual inspection of the GUS and luciferase data was performed and lines scored from 0 to 3 depending on the signal intensity and pattern of expression (data not shown). Surprisingly, almost all of the *VR-ACS1* promoter transgenic lines showed very strong expression of the reporter gene (either *gusA* or *luc*). Based on this data, 16 *VR-ACS1*:GUS, 8 *VR-ACS1*:LUC, 3 CaMV35S:GUS and 4 CaMV35S:LUC representative lines were grown and self pollinated to obtain T_2 homozygous plants. Quantitative analysis of leaf tissue from young homozygous T_2 seedlings confirmed the initial observations from the screening process showing high GUS and LUC activities. Figure 3 shows reporter gene activity values for seven independent *VR-ACS1*:GUS and *VR-ACS1*:LUC transgenic lines as well as two independent CaMV35S:GUS and CaMV35S:LUC transgenic lines. *VR-ACS1*:GUS lines consistently displayed high activity values that were, on average, four times higher than those observed for CaMV35S:-GUS transformants. Luciferase activity values were not as uniform as in *VR-ACS1*:GUS transformants but were consistently higher than CaMV35S:LUC lines. Based on these results, several homozygous *VR-ACS1*:GUS (2.5G#3-4, #7-3, #10-3), *VR-ACS1*:LUC (2.5L#4-10, #25-1), CaMV35S:GUS (35SG#5-2, #11-3) and CaMV35S:LUC (35SL#10-6, #26-7) transgenic lines were chosen for further analysis. Southern and segregation analyses revealed the presence of single copies of the transgene in each of the lines except for 35SG#5-2, which contained two copies (data not shown).

The selected transgenic lines were qualitatively and quantitatively analyzed for reporter gene expression at different developmental stages. Figure 4a–j shows a representative sample of GUS and LUC assays performed during seed germination, early seedling development and different tissues from mature plants of transgenic tobacco lines carrying *VR-ACS1* promoter constructs. Strong, constitutive reporter gene expression was observed in each of the transgenic lines. Intense GUS staining was observed in roots, cotyledons, leaves and hypocotyls at all stages of seedling

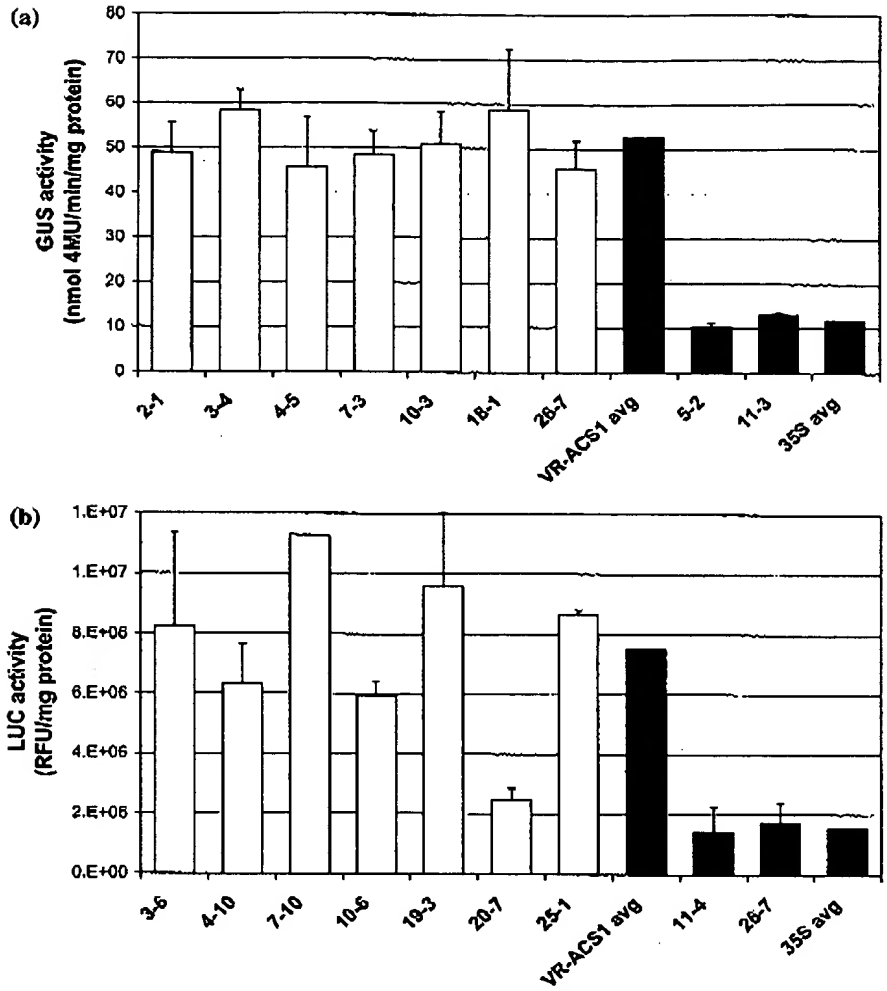

Figure 3. Preliminary screening of T_2 homozygous tobacco lines. Reporter gene expression in representative *VR-ACS1* and CaMV 35S promoter transgenic lines. First and second true leaves from 20-day-old transgenic tobacco plants grown in a growth chamber were pooled and assayed in duplicate. GUS activities are expressed in nmoles 4MU/min/mg of soluble protein. LUC activities are expressed in relative light units/mg of soluble protein. Error bars represent standard error. Dark bars represent the average for all *VR-ACS1* and 35S promoter lines (as labeled) assayed. (a) GUS activities of seven independent *VR-ACS1*:GUS transgenic lines (2.5G#2-1, #3-4, #4-5, #7-3, #10-3, #18-1, #26-7) and two CaMV35S:GUS (35SG#5-2, #11-3) transgenic lines. (b) Luciferase activities of seven independent *VR-ACS1*:LUC transgenic lines (2.5L#3-6, #4-10, #7-10, #10-6, #19-3, #20-7, #25-1) and two CaMV35S:LUC (35SL#11-4, #26-7) transgenic lines.

development (Figure 4a, b). In mature plants, strong, non-specific GUS expression was observed in most tissues, including the epidermis, cortex, pith, xylem, phloem, sepals, stigma and anthers (Figure 4c–h). GUS staining was not consistently observed in root tips, root hairs or trichomes in both *VR-ACS1*:GUS and CaMV35S:GUS seedlings (data not shown). Luciferase *in vivo* assays confirmed the GUS observations with the *VR-ACS1* promoter directing strong expression in all tissues of tobacco seedlings (Figure 4i). For comparison purposes, a CaMV35S:LUC plant is shown in Figure 4j. The false color image clearly demonstrates that the CaMV35S:LUC plant exhibits lower

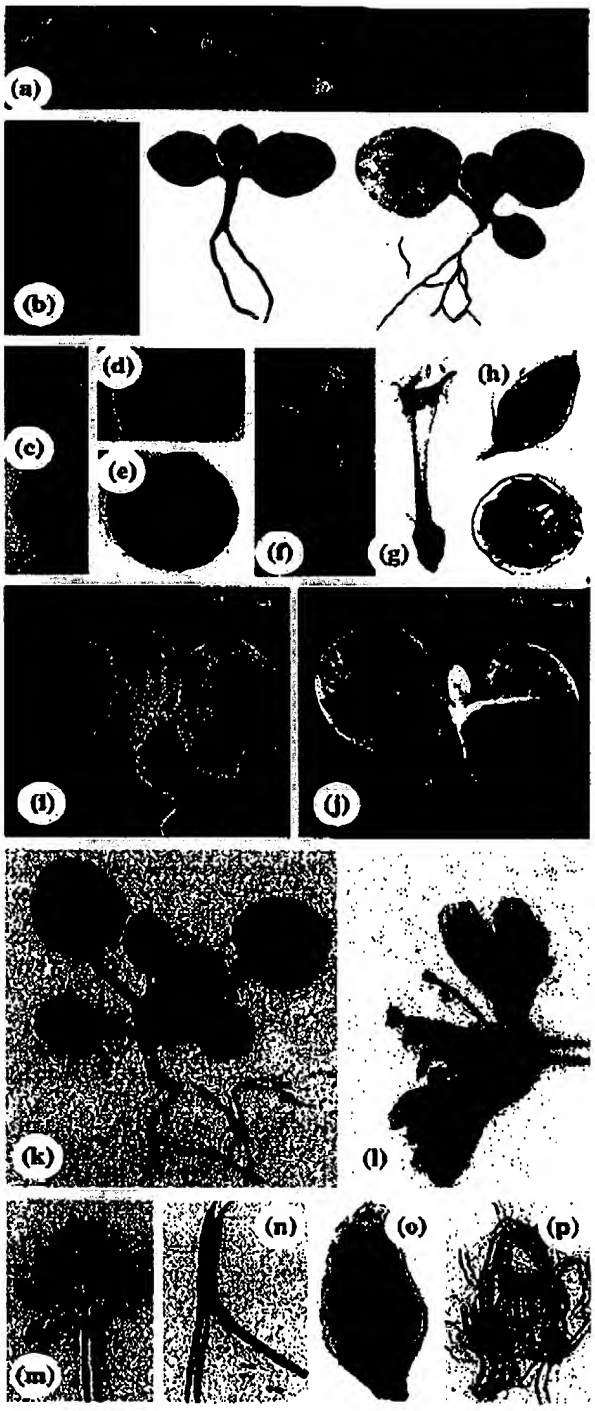
(a)
(b)
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Figure 4. Histochemical analysis of GUS and luciferase expression in transgenic tobacco and *Arabidopsis* plants. Transgenic tobacco plants carrying the full length *VR-ACS1* promoter fused to the GUS (a–h) or luciferase (i) reporter genes were assayed as described in materials and methods. (a) germinating seeds at emergence, root and hypocotyl extension after seed germination; (b) 6, 12 and 18-day-old seedlings; (c) transverse sections of a stem with a developing petiole (up) and a petiole (down); (d) transverse section of stem; (e) transverse section of root; (f) floral meristem; (g) fully expanded flower; (h) seed pods; (i, j) *In vivo* luciferase assays on 18-day-old seedlings from a *VR-ACS1*:LUC and a CaMV35S:LUC lines. The images are shown in artificial color corresponding to the intensity of light emission. Color bar scales above the images represent the minimum and maximum values set to each color, with black denoting no expression and white color representing maximum expression. Note that the scales for *VR-ACS1* and CaMV 35S promoter plants are different (max for *VR-ACS1* is 569, max for CaMV 35S is 169). (k–p) Histochemical staining of transgenic *Arabidopsis* plants carrying the full length *VR-ACS1* promoter fused to GUS. (k) 10-day-old seedling; (l) open flower; (m) inflorescence; (n) mature siliques (excised to show seeds); (o) cauline leaf; (p) roots.

levels of light emission, even though the maximum value of the scale (169 corresponding to white color) was set substantially lower than the maximum for the *VR-ACS1* promoter line (579 corresponding to white color).

Quantitative GUS and LUC activity assays of *VR-ACS1* and CaMV 35S promoter transgenic lines were performed in several tissues from immature tobacco plants (approximately 20 days old and 10 cm tall) grown in an environmentally controlled growth chamber. Figure 5 shows the GUS and LUC activities expressed in units per mg of total soluble protein and similar trends of expression were observed when the activity values were referred to fresh weight (data not shown). GUS activity values in the two *VR-ACS1*:GUS lines analyzed were almost identical in all the tissues examined and consistently higher than the CaMV35S:GUS line (Figure 5a). Again, luciferase activity results exhibited more variability than GUS but showed consistently higher levels of activity in the *VR-ACS1* promoter lines (Figure 5b). Young and rapidly developing tissues showed the highest expression levels for both promoters in immature tobacco. Average GUS and LUC activities were calculated showing approximately 4 times higher values for the *VR-ACS1* promoter lines when compared to the CaMV 35S lines (Figure 5).

GUS gene expression was analyzed in mature tobacco plants at vegetative and flowering stages of development (Figure 6). Fluorometric assay of GUS activity in mature, non-flowering plants shows that the two *VR-ACS1* promoter lines analyzed display similar levels that are consistently higher than those observed for the CaMV 35S representative line (Figure 6a). As observed in immature plants, the organ-specific trend of GUS activity in *VR-ACS1* lines is parallel to that observed for the CaMV 35S promoter, with stronger expression being observed in leaves, petioles and stems, and somewhat lower levels in roots. When activity levels are referred to fresh weight, a different trend is observed; immature leaves exhibit the highest levels of activity, which subsequently decrease as the leaf matures and starts to senesce (results not shown). Very similar results were observed when flowering tobacco plants were analyzed (Figure 6b) with roots, petals and sepals showing the lowest levels of activity. As was observed in the non-flowering plants, higher GUS activity was consistently observed in *VR-ACS1* lines versus CaMV 35S lines. Average activity values were again 4–6 times higher for the *VR-ACS1* promoter lines.

To determine if the results observed in transgenic tobacco plants were due solely to the effect of the *VR-ACS1* promoter or whether any genetic element present in the T-DNA could be influencing the strength of the observed *VR-ACS1*-driven expression, we built a new set of promoter–reporter gene fusions using the pBluescript plasmid. The pVRACS1–iGUS construct contains the full length *VR-ACS1* promoter including the 5′UTR fused to the intron-containing GUS reporter gene and nopaline synthase terminator sequence (NosT) (Tanaka et al., 1990). p35SPAE–iGUS harbors the CaMV 35S promoter, up to the start of transcription, fused to the 5′UTR of the pectinacetylesterase gene (PAE) from *Vigna radiata* (Breton et al., 1996), followed by the intron-GUS gene and NosT. The PAE 5′UTR was trimmed to 85 bp in order to have identical lengths of the 5′UTRs in both constructs. Transient assays were performed in tobacco cell cultures by co-bombarding with an equal mixture of DNA containing either pVRACS1–iGUS or p35SPAE–iGUS and p35SLuNt constructs. p35SLuNT carries the firefly luciferase reporter gene and enabled normalization of the quantitative GUS fluorometric assays. The normalized GUS activity data obtained from the transient

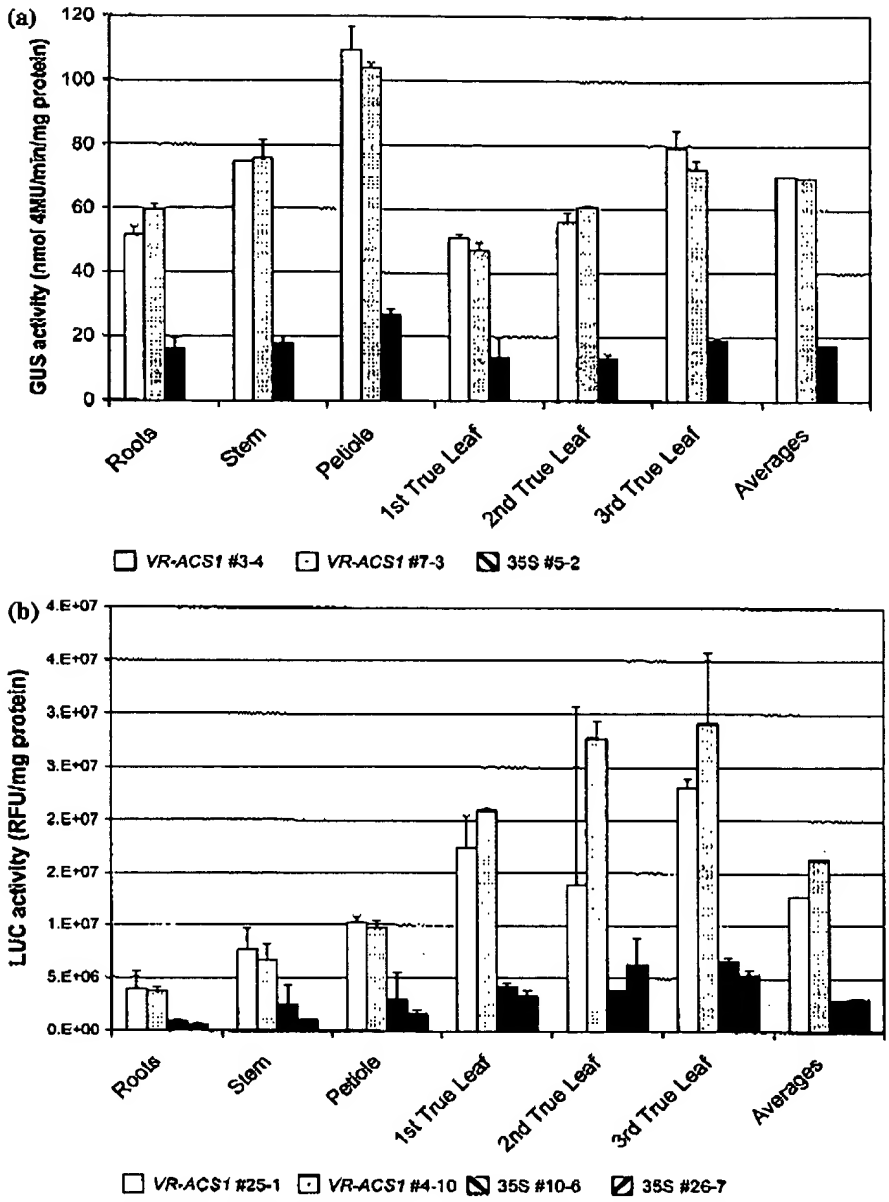


Figure 5. GUS and LUC expression in different tissues of immature *VR-ACS1* and CaMV 35S promoter transgenic tobacco plants. Plants were randomly arranged in a growth chamber and grown at 25°C and 70% humidity for 28 days. Roots, stem (including the apical meristem), petioles and leaves from plants at a similar developmental stage, were assayed in duplicate. GUS activities are expressed in nmoles 4 MU/min/mg of soluble protein. LUC activities are expressed in relative light units/mg of soluble protein. Error bars represent standard error. The last position in each graph represents the overall average for each line. (a) GUS activity levels of two *VR-ACS1*:GUS lines (2.5G#3-4, #7-3) and one CaMV35S:GUS (35SG#5-2) line. (b) LUC activity levels of two *VR-ACS1*:LUC lines (2.5L#25-1, #4-10) and two CaMV35S:LUC (35SL#10-6, #26-7) lines.

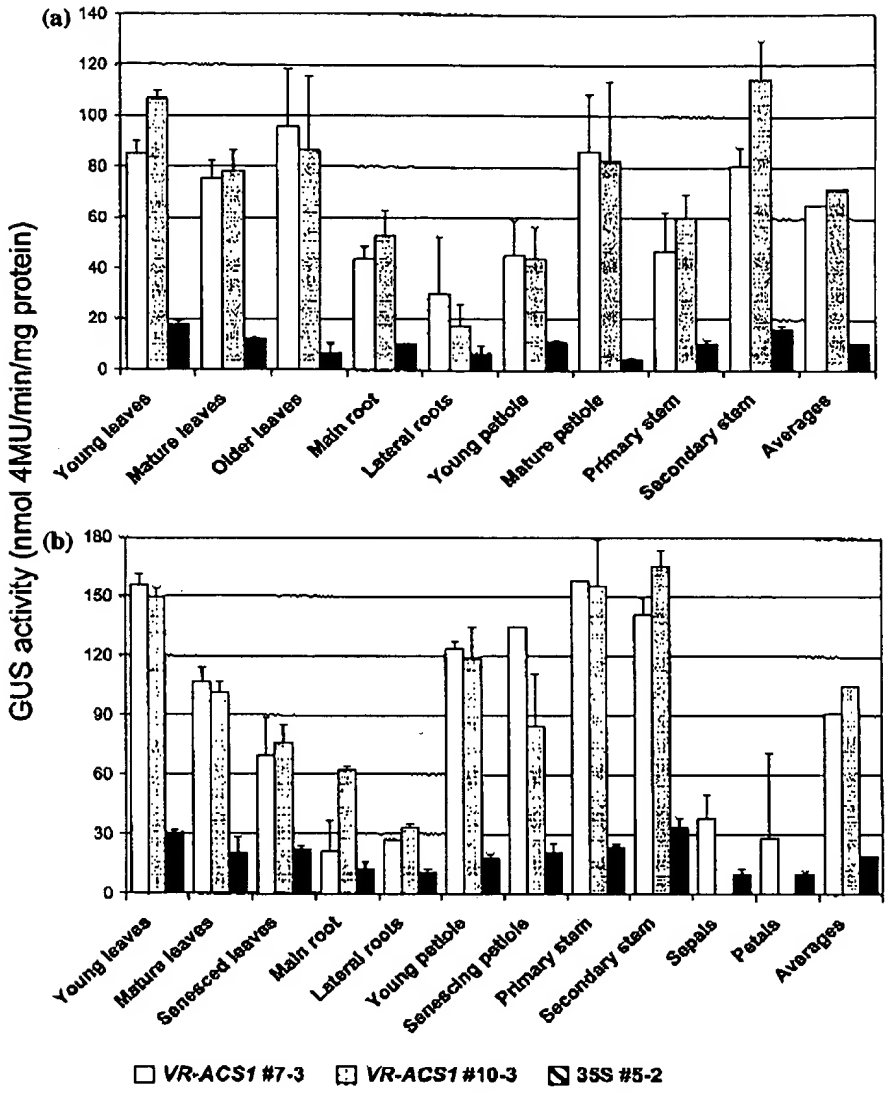


Figure 6. GUS expression in mature vegetative and flowering *VR-ACS1* and CaMV 35S promoter transgenic tobacco plants. Plants from two independent *VR-ACS1* lines (2.5G#7-3, #10-3) and one CaMV 35S line (35SG#5-2) were randomly arranged and grown in a glasshouse. Tissue samples from tobacco plants growing at a similar developmental stage were assayed in duplicate for GUS activity. Error bars represent standard error. GUS activities are expressed in nmoles 4MU/min/mg of soluble protein. The last position in each graph represents the overall average for each line. (a) Mature vegetative transformants at a similar developmental stage (12 to 15 cm high, 7–8 leaves). (b) Mature flowering transformants with fully open flowers. No assays were performed on sepal and petal tissues of line 2.5G#10-3.

assay experiments is consistent with our transgenic analysis and shows approximately 4 times higher activity levels for *VR-ACS1* promoter constructs relative to the CaMV 35S promoter (results not shown).

Transcription levels in VR-ACS1:GUS plants do not correlate with activity levels

Preliminary northern analysis performed on transgenic tobacco plants revealed that the levels

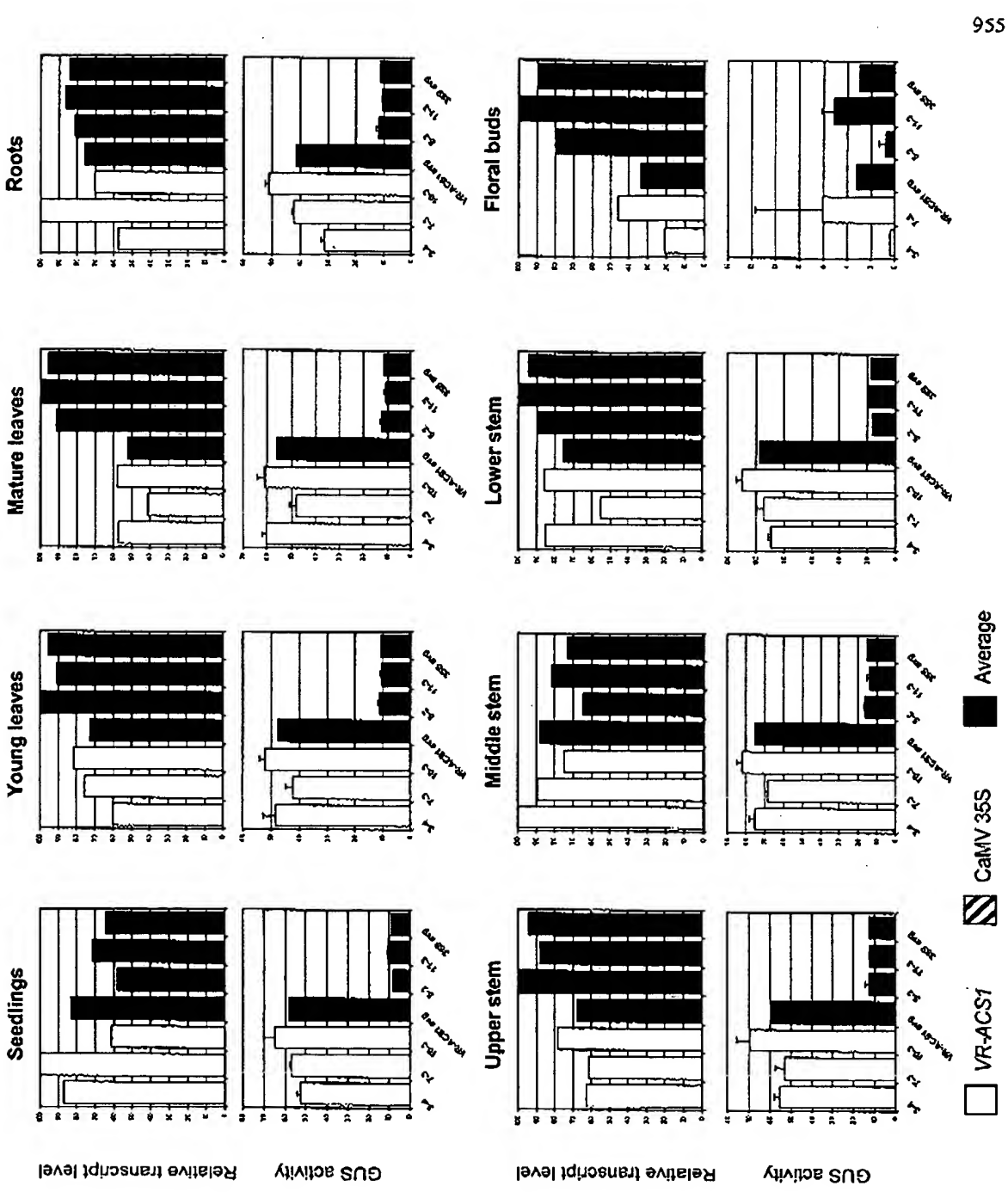
of GUS mRNA present in *VR-ACS1* plants were not several times higher than those of CaMV 35S transformants, as would be expected from enzyme activity measures (results not shown). To determine whether this was an isolated result or this was in fact common to all transgenic lines, we performed an experiment in which plants from three independent *VR-ACS1*:GUS transgenic lines and two CaMV35S:GUS lines were grown in a randomized plot and tissue collected simultaneously. For each of the transgenic lines, tissue was pooled from a number of plants, mixed and divided into two equal lots that were subsequently used to determine GUS mRNA and activity levels. The results are shown in Figure 7. As observed repeatedly in our previous analyses, GUS activity levels were relatively consistent between all three *VR-ACS1* lines analyzed and several times higher than the activity levels exhibited by the two CaMV 35S lines analyzed (except for floral buds). The higher activity levels measured correspond to a higher relative abundance of GUS protein in the *VR-ACS1* promoter lines detected by western blots using antibodies raised against the GUS polypeptide (results not shown). GUS mRNA levels in *VR-ACS1* and CaMV 35S promoter lines were measured by northern analysis and quantified using a phosphoimager to compare intensity values. Total intensity values were normalized using a ribosomal probe to account for loading differences in the gel lanes and referred to as a percentage of the highest value obtained (Figure 7). These results show a striking difference between protein activity values and mRNA transcript levels. In contrast to the pattern of GUS activity levels observed, *VR-ACS1* and CaMV 35S lines showed similar levels of GUS transcript. Seedlings and middle stems show slightly higher transcript levels in *VR-ACS1* promoter lines, while young leaves, roots, upper and lower stems showed slightly higher levels in CaMV 35S lines. In mature leaves and floral buds the differences were more pronounced, with CaMV 35S lines containing more than twice the level of GUS transcript than *VR-ACS1* promoter lines. A parallel experiment was conducted, simultaneously comparing luciferase activity and mRNA levels in several tissues of one *VR-ACS1*:LUC and one CaMV35S:LUC line with similar results. Again, luciferase activity was always several times higher in *VR-ACS1* promoter tissues, whereas luciferase mRNA levels were similar in *VR-ACS1* and CaMV 35S tissues (results not shown).

Figure 7. Simultaneous analysis of GUS mRNA and activity levels in different tissues of *VR-ACS1* and CaMV 35S promoter tobacco lines. 14-day-old seedlings and tissues from mature tobacco plants with unopened floral buds were harvested from three independent *VR-ACS1* (2.5G#3-4, #7-3, #10-3) and two CaMV 35S promoter lines (35SG#5-2, #11-3) grown in a randomized plot, immediately frozen, macerated and thoroughly mixed. GUS mRNA transcript (upper graph) and protein activity (lower graph) levels from each tissue were measured by northern analysis and fluorometric GUS assays, respectively. Dark bars represent the average for all *VR-ACS1* and CaMV 35S promoter lines (as labeled) assayed. Northerns were first hybridized with a GUS probe and subsequently the probe stripped off the membranes and re-hybridized to a ribosomal probe. Signals were quantified using a phosphoimager and normalized against the ribosomal probes. An arbitrary value of 100 was given to the maximum value and the rest expressed as a percentage of the maximum. GUS activities are expressed in nmoles 4 MU/min/mg of soluble protein.

Deletion analysis of the VR-ACS1 promoter in tobacco

A series of five 5′ deletions of the *VR-ACS1* promoter, −1032, −791, −611, −366 and −141, were fused to the GUS reporter gene (Figure 2). A minimum of 10 independent T_0 tobacco lines were produced for each deletion construct and histochemically stained for GUS activity during seedling development (data not shown). Strong, constitutive GUS expression was observed throughout development for the majority of all five *VR-ACS1* promoter deletions.

Plants from a representative homozygous transgenic line for each *VR-ACS1* deletion were grown to maturity and GUS activity determined in different tissues (Figure 8). In general, there was strong, constitutive reporter gene expression in all organs assayed for each of the *VR-ACS1* deletions. All tissues analyzed, with some exceptions, displayed similar GUS activities for each of the *VR-ACS1* promoter deletions and the values were consistent with the data previously obtained for the full length (2.5 kb) *VR-ACS1* promoter (Figures 5–8). In leaves, there was a drop in gene expression observed in the −366 and −141 deletions suggesting the presence of a leaf-specific *cis*-element in the −611 to −366 region. Similarly,


Roots
Floral buds
Mature leaves
Lower stem
Young leaves
Middle stem
Seedlings
Upper stem
GUS activity
Relative transcript level
VR-ACS1
CaMV 35S
Average

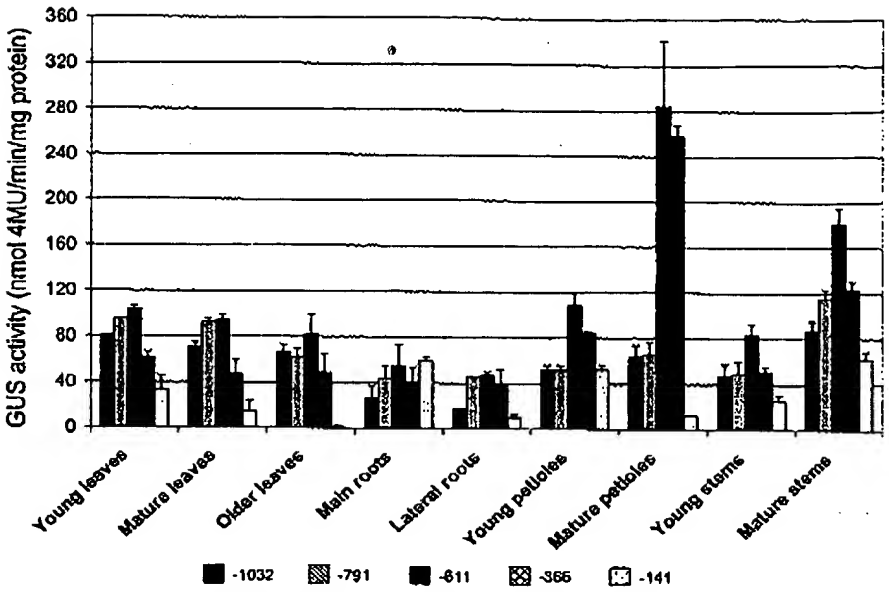


Figure 8. Analysis of *VR-ACS1* promoter deletions in tobacco. GUS expression levels in tissues of homozygous tobacco lines carrying different *VR-ACS1* deletion constructs. Deletion lines shown in this figure include −1032 (#7), −791 (#9), −611 (#10), −366 (#3) and −141 (#5). Tissues from mature vegetative transformants at a similar developmental stage (12–15 cm high, 7–8 leaves) were assayed for GUS activity and expressed in nmoles 4 MU/min/mg of soluble protein. Error bars show the standard error.

there is a marked drop in activity observed in lateral roots for the −141 deletion which could indicate the presence of a specific element in the −366 to −141 region. Unusually high levels of activity were detected in the −611 and −366 deletions in mature petiole tissues while relatively normal levels were detected in young petiole tissues. The general trend of GUS activity among the different tissues resembles that previously observed for the full length *VR-ACS1* promoter (Figures 5–6). Only a single deletion line was quantitatively assayed for GUS activity, and therefore analysis of more deletion lines is necessary in order to confirm the tissue specific activity levels observed. Considering that the promoter drives undetectable levels of *VR-ACS1* gene expression in mung bean under normal physiological conditions, it was remarkable to find that even the smallest *VR-ACS1* promoter deletion consisting of −141 bp plus the 5′UTR showed strong constitutive expression. These results suggest that there are strong transcriptional enhancer elements present in the −141 deletion, either in the core promoter region or the 5′UTR, which can drive strong expression levels similar to or even in excess of the CaMV 35S promoter. Alternatively, as suggested by the results shown in Figure 10, the overall activity of the −141 deletion could be due to translational enhancer activity.

Developmental characterization and deletion analysis of the VR-ACS1 promoter in Arabidopsis

The striking results obtained during the characterization of the *VR-ACS1* promoter in tobacco prompted us to study a different plant species in order to determine whether the high expression levels observed are restricted to tobacco. For this purpose, the *VR-ACS1*:GUS binary construct containing the full length *VR-ACS1* promoter fused to the GUS reporter gene was used to produce transgenic *Arabidopsis thaliana* lines. As with tobacco, a large number of independent lines were produced and subjected to a preliminary screen to select representative lines. The first cauline leaf from 38 independent T_1 transformants was stained for GUS activity (results not shown). Most lines displayed strong GUS staining and T_2 homozygous individuals were obtained from several representative lines for further analysis.

Transgenic *Arabidopsis* lines carrying the CaMV35S:GUS construct were produced and screened in a similar manner.

Histochemical GUS assays were performed on seedlings as well as a number of tissues from mature plants (Figure 4 k–p). Strong staining was observed in leaves, petioles and roots of 10-day-old seedlings, while stems showed a more irregular staining pattern that could indicate substrate penetration problems (Figure 4k). In mature plants, intense staining was observed throughout the root system (Figure 4p) as well as in the petals and sepals of flowers (Figure 4l). Floral reproductive organs such as filaments, anthers, stigma, style and ovary also showed high levels of GUS activity (Figure 4l). Siliques and seeds showed strong staining (Figure 4n). In leaves, the staining was particularly intense in the vascular tissues of the blade and the petiole (Figure 4o). GUS activity results were quantified in different tissues from two *VR-ACS1* and two CaMV 35S promoter transgenic lines, and the results shown in Figure 9. As observed in tobacco, the full length *VR-ACS1* promoter shows a general trend of higher activity, between two to eight times higher, than the CaMV 35S promoter in seedlings and all tissues studied in mature *Arabidopsis* (Figure 9).

In addition to the full length *VR-ACS1* promoter, three 5′ deletions were studied in *Arabidopsis*, −1032, −366 and −141. A number of independent transgenic lines were obtained for each deletion construct and after initial characterization, two lines per deletion were selected to produce homozygous T_2 individuals. Unlike tobacco, where most deletions showed similar activity, the −366 deletion construct exhibited very high activity in *Arabidopsis*, on average twice that of the full length *VR-ACS1* promoter. A third independent line containing the −366 deletion was analyzed to confirm these results and was proven to have similar values to the two lines shown in Figure 9 (result not shown). The consistently high levels of activity observed in three independent lines makes it unlikely that these results are due to T-DNA insertion effects. Similar results were also observed in T_2 generation seedlings of other −366 lines, reinforcing our observations. There is no straight forward explanation for the high levels of activity directed by the −366 deletion construct, although it is interesting to note that several tandem and inverted repeat sequences present in the −1032 fragment are missing in this construct. GUS activity in lines of the −1032 deletion fell within the range of activity levels measured in the full length *VR-ACS1* promoter in all tissues with the exception of seedlings where line −1032#11-24 reproducibly showed very high levels of activity, comparable to the levels observed in −366 lines. Nevertheless, line −1032#11-24 did not show these high levels in any of the mature tissues, thus the activity observed in seedlings could be related to some kind of alternative regulation of the chromosomal region where the T-DNA is inserted. The smallest deletion construct studied (−141), showed levels of activity equivalent to or slightly lower than the full length *VR-ACS1* promoter in all tissues studied, though the levels observed were generally higher than the CaMV 35S promoter. Thus, even the smallest *VR-ACS1* promoter deletion is capable of directing strong, constitutive promoter activity in *Arabidopsis*. This result confirms previous findings in tobacco and supports the hypothesis that the activity of the *VR-ACS1* promoter is due to the presence of strong 'transcriptional enhancer elements' and/or enhanced translation initiation from the region downstream of −141.

Keeping in mind the disparity between protein activity levels and mRNA levels observed in *VR-ACS1* and CaMV 35S promoter transgenic tobacco lines, it was important to establish whether a similar phenomenon could be observed in *Arabidopsis*. For this purpose we performed simultaneous GUS activity and northern analyses in seedlings of two lines for the full length promoter, one line each for the −1032, −366 and −141 deletions and two CaMV 35S lines (Figure 10a). Comparison of specific GUS mRNA levels showed that *VR-ACS1* promoter lines contained between 35 and 85% of the levels found in CaMV 35S lines, but GUS activity values were vastly superior in *VR-ACS1* lines (between 2 and 8 times higher) despite the lower transcript levels. The −366 line displayed the highest transcript levels (~85% of the CaMV 35S levels), which is in agreement with the higher activity levels observed for this construct (Figures 9 and 10a). Simultaneous mRNA and activity measurements were also performed on different tissues of mature plants for one full length *VR-ACS1* promoter line and one CaMV 35S line (Figure 10b). Again, transcript levels driven by the *VR-ACS1* promoter were

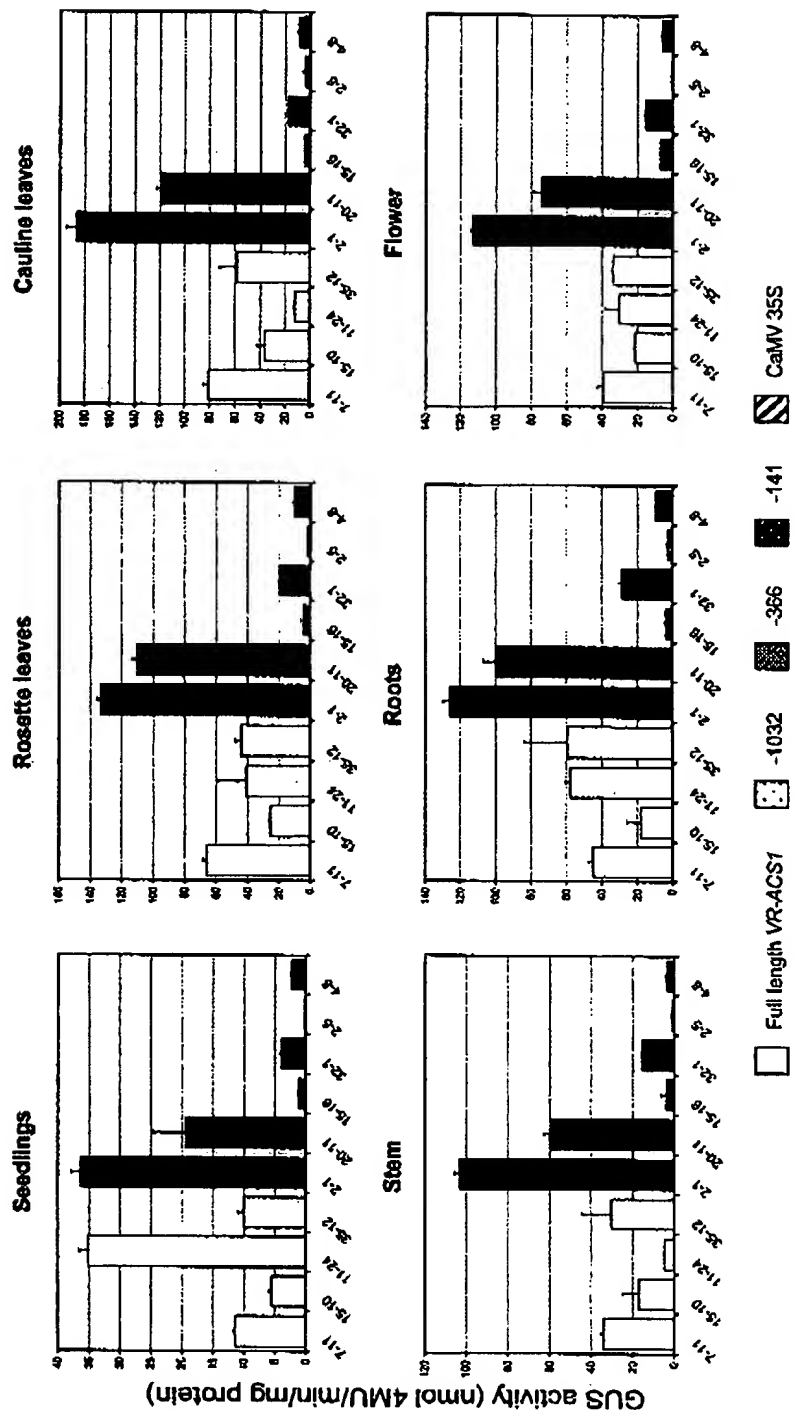

Cauline leaves
Flower
Rosette leaves
Roots
Seedlings
Stem
GUS activity (nmol 4MU/min/mg protein)
Full length VR-ACS1
-1032
-366
-141
CaMV 35S

Figure 9. GUS expression analysis of *VR-ACS1* full length and selected deletions in *Arabidopsis*. Tissue was harvested from mature 7-week-old *Arabidopsis* plants of two independent lines (#7-11, #15-10) carrying the full length *VR-ACS1* promoter and −1032 (#11-24, #35-12), −366 (#2-1, #20-11) and −141 (#15-16, #32-1) deletion lines, and assayed for GUS activity in duplicate. Two independent CaMV 35S lines (#2-5, #4-8) were included in the analysis for comparisons. Error bars represent standard error. GUS activities are expressed in nmoles 4 MU/min/mg of soluble protein.

lower; between 48 and 93% of those measured in CaMV 35S plants, while GUS activity was 4–6 times higher in *VR-ACS1* tissues. These results were consistently observed across various developmental stages and tissues of mature *Arabidopsis* plants. Therefore, the discrepancies observed in tobacco between protein activity and mRNA transcript levels are reproducible in *Arabidopsis*.

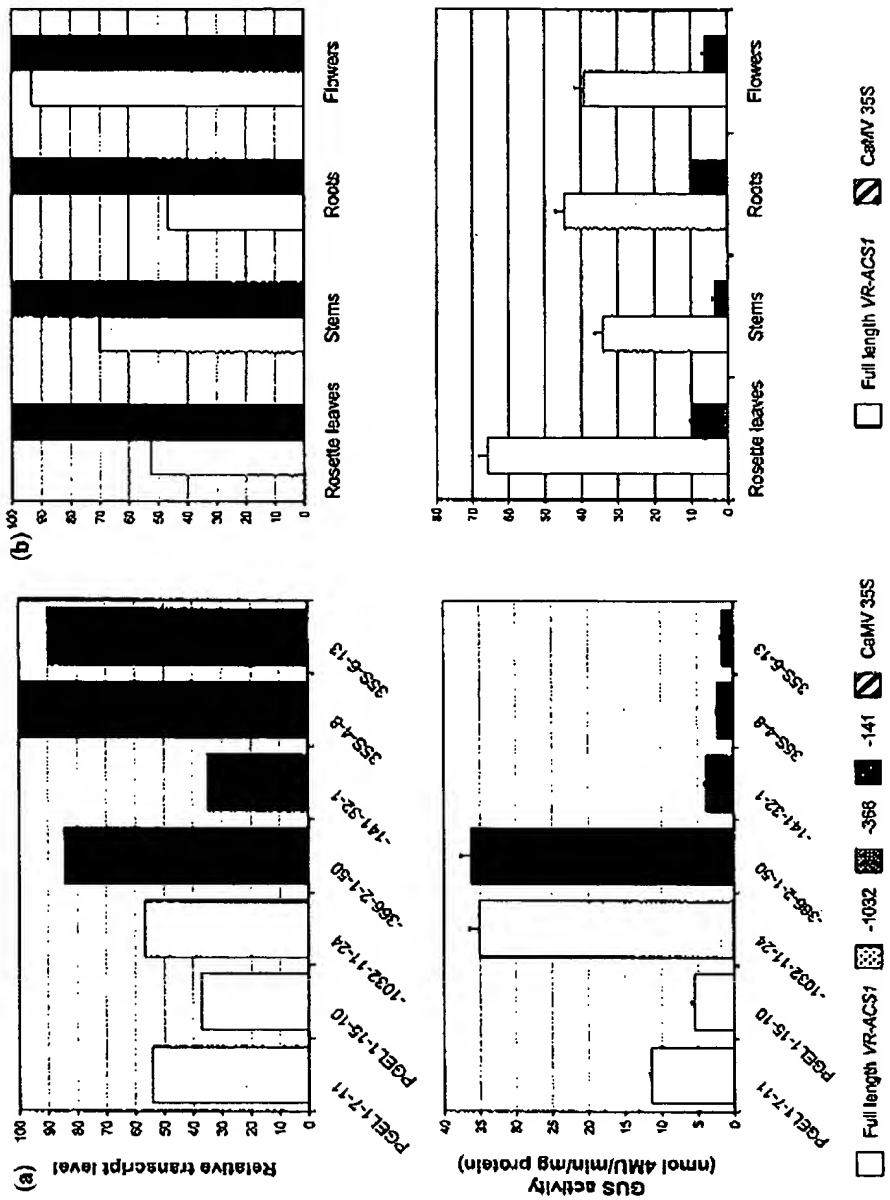
VR-ACS1 promoter activity in tobacco is unaffected by stimuli inducing VR-ACS1 in mung bean

Even though the constitutive behavior of the *VR-ACS1* promoter in tobacco and *Arabidopsis* clearly differs from that of the native gene in mung bean, we wanted to determine if the inducible nature of the promoter in its original system would be conserved in a heterologous host. For this purpose, the effect of auxin, NaCl and cycloheximide (CHX) on GUS expression driven by the *VR-ACS1* promoter was examined in tobacco leaves. Transgenic tobacco plants carrying the full length *VR-ACS1*:GUS construct were grown in a glasshouse without any mechanical stimulation. Leaf discs from mature leaves were collected and incubated in distilled water, 250 μM IAA, 100 mM NaCl, or 50 μM cycloheximide for 3 hours. Figure 11 shows the effects of the different treatments on *VR-ACS1* driven GUS mRNA expression and protein activity. The results show that there is no clear induction in either GUS transcript levels or protein activity by any of the treatments. The basal GUS transcript and activity levels in the water control treatment at times 0 and 3 h were very high as routinely observed in all tissues studied. The lack of further induction by any of the treatments could be interpreted as though the promoter is completely de-regulated in tobacco, achieving its maximum possible activity.

Discussion

We have previously cloned and characterized a mung bean ACC synthase cDNA from a gene (initially named *AIM-1* and later renamed *VR-ACS1* to comply with the guidelines for ACC synthase gene nomenclature) that displays very little or undetectable basal expression levels under normal growth conditions, as determined by northern and nuclear run-on experiments (Botella et al., 1992). However, *VR-ACS1* is rapidly and highly induced by a variety of physical and chemical stimuli (Botella et al., 1992). Among those stimuli are several hormones such as auxins, cytokinins and abscisic acid, several abiotic stresses (dehydration, salt), touch stimulation, cycloheximide treatment and heterotrimeric G-protein activators such as AlF_4 (Botella et al., 1992, 1995; Yi et al., 1999; Yoon et al., 1997 and unpublished results). In addition to these individual responses, some hormones such as auxins and cytokinins can have synergistic effects or negatively affect the action of another hormone (e.g. brassinosteroids can repress the induction of gene expression by auxins and cytokinins) (Botella et al., 1992, 1995; Yoon, et al., 1997; Yi, et al., 1999). The *VR-ACS1* gene is therefore a very good candidate to study the mechanisms by which so many signals can assert their regulatory action on a single gene. We were interested in determining whether a number of different *cis*-elements separately control the response to each individual stimulus or whether several stimuli share *cis*-elements and/or *trans*-factors to explain the complex effects observed following hormone and stress treatments.

The strong histochemical staining observed in tobacco and *Arabidopsis* transgenic lines containing the *VR-ACS1* promoter fused to the GUS reporter gene was very surprising given that native *VR-ACS1* mRNA levels in mung bean are almost undetectable under non-inducing conditions. There is no simple explanation for the expression levels observed in *VR-ACS1*:GUS transformants. Analysis of the *VR-ACS1* promoter sequence revealed a number of previously described consensus sequences for *cis*-elements involved in auxin, ABA and dehydration response. Nevertheless, it is clear that the *VR-ACS1* promoter, when taken out of its original host, directs high levels of expression in all tissues



assayed in two different plant species. In contrast, the promoters of two other auxin-induced ACC synthase genes from mung bean, *VR-ACS6* and *VR-ACS7*, were active under conditions shown to induce native gene expression when tested in the same two heterologous systems studied here (Yi et al., 1999; Yoon et al., 1999; Joo et al., 2004). Both promoters directed a marked increase in GUS reporter gene expression in response to induction by various stimuli, including hormones such as auxin and brassinosteroids, either acting alone or in synergism. It is interesting to note that while *VR-ACS1*, *6* and *7* are the only auxin-inducible ACC synthase genes identified in

Figure 10. Simultaneous analysis of GUS mRNA and activity levels in different tissues of *VR-ACS1* and CaMV 35S promoter *Arabidopsis* lines. Plants were grown simultaneously under identical conditions and tissues collected and immediately frozen. Frozen material was ground and thoroughly mixed before determining GUS activity and transcript levels. GUS activity measurements are expressed in nmoles 4 MU/min/mg of soluble protein. Error bars represent the standard error. To measure transcript levels 10 μg of total RNA was separated in agarose gels, transferred to nylon membranes, hybridized with a GUS probe and exposed to phosphoimager screens. After stripping the GUS probe, the membranes were re-hybridized using a ribosomal probe, exposed to phosphoimager screens and the values used for normalization. The maximum value was given an arbitrary value of 100 and the remaining expressed as a percentage of the maximum. (a) GUS activity and transcript levels in 14-day-old seedlings of two full length lines (#7-11, #15-10), two CaMV 35S (#4-8, #6-13) and one each of the -1032 (#11-24), −366 (#2-1) and −141 deletions (#32-1). (b) GUS activity and transcript levels in different tissues of a mature 7-week-old full length *VR-ACS1* (#7-11) and a CaMV 35S (#4-8) line.

mung bean, of these, only *VR-ACS1* expression is induced by the protein synthesis inhibitor, cycloheximide (Botella et al., 1992; Botella et al., 1995).

Our unexpected results made us review cloning and analysis strategies to confirm the lack of any technical artifacts. A number of factors can affect the overall activity of a promoter construct when studied in transgenic plants. One of them is the T-DNA structure of the binary vector used for plant transformation, pPZP111. The promoter–reporter gene junctions of all constructs have been sequenced to eliminate the possibility of cloning artifacts. The cloning strategy of the *VR-ACS1* promoter:reporter gene cassette into the T-DNA of the binary plasmid was carefully planned. The *VR-ACS1*:reporter gene cassettes were always cloned in opposite orientation to the selectable marker gene to avoid read through activity from the CaMV 35S promoter which drives the marker gene. In addition, the *VR-ACS1*:reporter cassettes were cloned into the T-DNA with the *VR-ACS1* promoter distal from the insertion border to avoid enhancer effects from genomic sequences close to the integration site. Nevertheless, CaMV 35S enhancers can act in either orientation, therefore it is impossible to discard an indirect effect of these enhancers in the *VR-ACS1*-driven expression in pPZP111. The best way to discard the existence of unforeseen T-DNA generated artifacts is to use a completely different vector. In our case we chose to use the pBluescript plasmid as carrier and built new promoter:reporter constructs separately from any of the constructs previously used for the stable transformation experiments. We also used a different assay system (transient vs. stable transformation) and even the source of the reporter gene was different (intron-GUS vs. 'intronless' GUS). The results observed in the transient transformation assays of tobacco cell cultures were very consistent with the stable transformation results. Using the new promoter:reporter gene constructs, normalized GUS activity values driven by the *VR-ACS1* promoter were approximately 4.5-fold higher than the values observed for the CaMV 35S promoter. In addition to this, a third assay method using different constructs was also tested. Using a *recently developed Agrobacterium*-mediated transient assay system to quantify promoter-luciferase reporter gene fusions in tobacco leaf tissues, we observed strong *VR-ACS1* promoter expression levels (relative to CaMV 35S) (results not shown). In summary, we have used

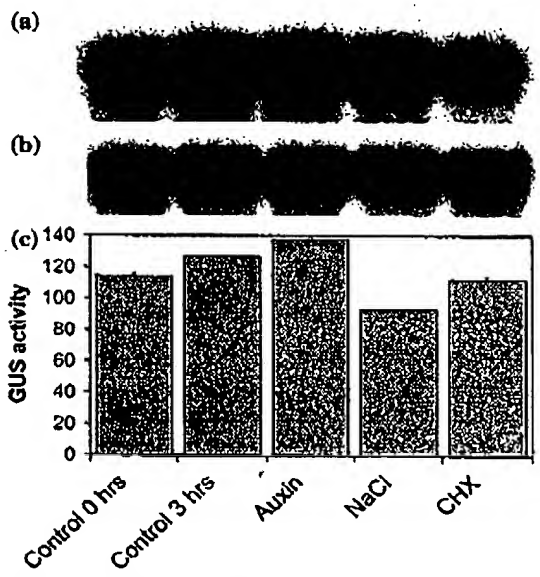


Figure 11. Inducibility studies of *VR-ACS1* in tobacco. Leaf discs from a full length *VR-ACS1*:GUS transformant (#7-3) were floated in either water, 250 μM IAA, 100 mM NaCl, or 50 μM cycloheximide for 3 h. After the treatments, tissue was harvested, ground in liquid nitrogen and assayed for GUS activity and mRNA levels. (a) Northern analysis of 10 μg of RNA hybridized with a GUS probe. (b) Membranes used in (a) were stripped and re-hybridized with a ribosomal probe. (c) GUS activity expressed in nmoles of 4 MU/min/mg of soluble protein.

three different vector systems with different vector backbones and consistently observed strong *VR-ACS1*-reporter gene activity in tobacco tissues. We can therefore safely conclude that the results observed in our stably transformed lines are not an artifact due to the transformation vector used.

Another important parameter that can affect expression patterns is the site of integration of the transgene. It is therefore imperative to produce sufficient numbers of independent transgenic lines and perform a preliminary screening of primary transformants in order to determine a consensus expression pattern and select representative lines for further characterization. We consistently analyzed a large number of primary transformants before selecting the lines to be used for promoter–reporter gene analysis. As an example, 32 lines were produced for the 2.5 kb *VR-ACS1*:GUS construct and 39 for the CaMV35S:GUS construct in tobacco. Our analysis of primary transformants revealed that most lines showed very similar expression levels and consistent expression patterns, even though a few lines of each construct showed variable staining intensities and patterns that deviated considerably from the consensus. The results observed in primary transformants were confirmed by more precise quantitative GUS activity assays performed in homozygous individuals from several lines. The use of luciferase as a second reporter gene strengthens the results obtained with GUS and provides a very coherent picture. As with GUS reporter gene lines, a large number of LUC primary transformants were analyzed to select representative lines (37 lines for the 2.5 kb *VR-ACS1*:LUC construct and 35 for the CaMV35S:LUC construct). Again, the vast majority of the *VR-ACS1*:LUC lines showed stronger light emission than the CaMV 35S lines. Qualitative *in situ* analysis was confirmed by quantitative *in vitro* assays on homozygous individuals, which showed higher levels of activity in *VR-ACS1* versus CaMV 35S promoter lines. In total we have analyzed, using either histochemical assays or activity measures in excess of 300 independent transgenic lines using two different reporter genes in two different species with consistent results. On average, the full length *VR-ACS1* promoter lines showed 4–6 times higher reporter gene activity than CaMV 35S lines.

Deletion analyses showed that even the smallest deletion (−141) containing the core promoter region (−141 bp plus +85 bp of the *VR-ACS1* untranslated leader sequence) was enough to confer high expression levels in tobacco. Expression levels were lower, though somewhat comparable to the full length promoter in most tissues. In *Arabidopsis*, the smallest region studied (−141) also resulted in strong expression, while the −366 deletion showed unusually high activity. From our data it is reasonable to hypothesize that there could be one or more strong promoter enhancer elements contained in the vicinity of the core promoter region of *VR-ACS1* or in the 5′UTR.

The *VR-ACS1* core regulatory domain contains putative consensus TATA and CAAT boxes. When −90 bp of the *VR-ACS1* core regulatory domain was compared to the equivalent proximal sequence of the CaMV 35S promoter (−1 to −90 bp), they shared 75% sequence homology upstream from and including the TATA box (Molina & Grotewold, 2005). Several discrete motifs such as a 6 nt (TTCCTC) and a 9 nt (TTCATTTCA) motif directly upstream of the TATA box and transcription start site, respectively, are perfectly conserved in both promoters.

It is clear from the tobacco and *Arabidopsis* studies that despite showing several times higher reporter activity levels, the *VR-ACS1* promoter lines contained transcript levels only comparable to or even lower than the CaMV 35S lines. The mRNAs resulting from the transcription of the *VR-ACS1* and CaMV 35S constructs are identical except for the 5′-untranslated regions, were the *VR-ACS1* construct incorporates the native *VR-ACS1* 5′UTR while the CaMV 35S construct used in this study contains a 5′UTR contained in the 'classic' pBI121 vector mostly comprised of non-genomic DNA arising from the cloning process (Herrera-Estrella et al., 1983; Sanders et al., 1987). The disparity between transcript and activity levels suggests that the *VR-ACS1* 5′UTR can enhance translation efficiency several fold in tobacco and *Arabidopsis*. There are a number of translational enhancers that have been shown to modulate protein accumulation in plants. Most of the naturally-occurring enhancers described to date, able to enhance translation several fold in tobacco plants, are present in viral leader sequences such as the Tobacco Mosaic Virus coat protein (TMV) and Satellite Tobacco Necrosis Virus coat protein (SNTV) (DeLoose et al., 1995). The most thoroughly characterized example from a plant origin

is the chlorophyll *a/b*-binding protein (Cab22L) leader sequence that can enhance translation several fold in transgenic tobacco plants (DeLoose et al., 1995). Whilst the UTRs of most plant genes are not well characterized, the UTRs of viruses have been extensively studied and shown to have a role in translation efficiency, transcript stability and pre-mRNA processing (Bolle et al., 1996, DeLoose et al., 1995). The 5'UTR of the *VR-ACS1* cDNA shares some general characteristics with other leader sequences that have been reported to exhibit strong translational enhancing activity (DeLoose et al., 1995). Like the 5'UTRs of *Cab22L* and the *TMV* viral coat protein genes, the *VR-ACS1* 5'UTR contains a low guanosine content which should reduce secondary structure formation and allow easier scanning for the first AUG in a favorable context. The *VR-ACS1* 5'UTR also contains CT rich sequences and poly CAA regions, which have been implicated in enhanced translation initiation in *Cab22L* and the *TMV* coat protein genes (DeLoose et al., 1995). We now have preliminary data indicating that the *VR-ACS1* 5'UTR enhances translation initiation to the same extent as the *Cab22L* gene leader sequence (manuscript in preparation).

The reasons for the unusually high levels of expression driven by the *VR-ACS1* promoter in tobacco and *Arabidopsis* are not evident. The *VR-ACS1* promoter was initially isolated with the intention of better understanding the mechanisms responsible for its interesting regulatory characteristics with an eye towards developing a promoter useful for biotechnological applications. *VR-ACS1*, like many other auxin-inducible genes, is highly inducible by cycloheximide, a protein synthesis inhibitor. At the start of our work there were two different hypotheses to explain this striking behavior. It was proposed that this class of auxin-inducible genes was under the control of a strong promoter that is continuously repressed by a short lived repressor protein. When tissues are treated with a protein synthesis inhibitor, repressor levels would rapidly fall and the gene would become de-repressed, resulting in the high levels of expression observed (Theologis et al., 1985). On the other hand it was also proposed that the action of cycloheximide might stabilize transcripts from auxin-inducible genes, therefore increasing their steady-state relative abundance (Franco et al., 1990). The two hypotheses are not mutually exclusive and a combination of both could occur simultaneously. The high levels of expression observed in *VR-ACS1* promoter transgenic plants could be explained if (a) the putative repressor(s) controlling expression of *VR-ACS1* in mung bean is not present in tobacco or (b) the orthologous tobacco repressor protein(s) does not recognize the *cis*-element(s) present in the *VR-ACS1* promoter. Either situation would result in de-repression and constitutive promoter activity. If this hypothesis proves true, a necessary corollary is that it should be possible to find other similar auxin/cycloheximide-inducible genes, providing a good source for new constitutive promoters. In addition, alternative explanations such as the existence in mung bean, but not in tobacco or *Arabidopsis*, of a transcriptional silencer either 5' (beyond the −2383 nucleotide) or 3' relative to the *VR-ACS1* promoter or the presence of *cis*-acting elements in the transcribed *VR-ACS1* sequences that regulate the mRNA stability under different conditions cannot be discarded.

More recently, a repressor/activator model has been proposed to explain the regulation of some auxin-responsive genes, although the mechanism is more complicated than the somewhat simplistic initial model described above. A number of *cis*-elements involved in auxin regulation of gene expression are known as auxin response elements (AuxRE). The better characterized AuxREs have the core sequence TGTCTC, which can bind a class of transcription factors known as auxin response factors (ARFs) (Ulmasov et al., 1997a). At least some of these ARFs, those with a Gln-rich middle domain, are transcriptional activators (Ulmasov et al., 1999). Aside from binding to the AuxRE promoter elements, ARFs can self dimerize or associate with another kind of nuclear proteins known as Aux/IAA proteins (Reed, 2001; Tiwari et al., 2003). According to the model proposed by Tiwari et al. (2003), when auxin content is below the activation threshold for a cell, ARFs are bound to AuxRE elements but are actively repressed by hetero-dimerization with the Aux/IAA proteins. When the auxin content rises, Aux/IAA proteins are targeted for rapid degradation by the proteosome pathway (Rogg & Bartel, 2001) and their depletion results in release of the ARFs, de-repressing transcription of the gene. Other transcription activating Gln-rich ARFs can then take the place vacated by the Aux/IAA

proteins and further boost gene expression (Tiwari et al., 2003).

The behavior of the *VR-ACS1* promoter in heterologous hosts cannot be explained by the above hypothesis, as it contains four putative AuxRE elements that would be expected to function independently of the plant species. Nevertheless, the model is by no means complete and does not necessarily apply to all auxin-induced genes, but only to primary response genes containing AuxRE elements with the TGTCTC core sequence (Hagen & Guilfoyle, 2002). In addition, a number of observations are not yet explained by the model, such as the fact that not all ARFs are activators and at least some of them act as repressors (Ulmasov et al., 1999). This is also the case for Aux/IAA proteins, which do not always repress gene expression; at least one has been shown to act as an activator of auxin-induced transcription (Leyser et al., 1996). Only a small subset of the ~22 ARF genes and the ~24 Aux/IAA genes present in *Arabidopsis* have been characterized in some detail, and therefore it seems inevitable that a more complex model will evolve as knowledge of the function of these proteins advances. In addition to the mechanism explained above, a number of variations are also possible in which additional elements or specific secondary structures in the promoter region can play important roles in the regulation of gene expression. The *VR-ACS1* promoter upstream regulatory domain contains an extensive array of *cis*-acting elements possibly involved in auxin, ABA, temperature and dehydration responses. It is not surprising to find these elements since the *VR-ACS1* gene is rapidly inducible by auxins (Botella et al., 1992), ABA (Kim et al., 1997b), salt and dehydration stress (Cazzonelli, unpublished data). Several inverted and direct repeats are also present in the *VR-ACS1* promoter. Two inverted repeat structures with greater then 75% identity are located in close proximity and are surrounded by AuxRE-, MYB-, and G-Box-like elements.

Even though it contains four putative AuxRE elements, the behavior of the *VR-ACS1* promoter is very atypical since it is not inducible by auxins in heterologous hosts. This is surprising since the presence of AuxRE sequences is sufficient to confer auxin inducibility even in artificial promoters (Ulmasov et al., 1997b). Studies of the auxin-inducible GH3 soybean promoter identified 3 AuxREs (Liu et al., 1994), two of which, D1 and D4, have been extensively characterized. A construct containing both elements fused to the minimal CaMV 35S had little effect on basal expression, yet conferred strong auxin-responsiveness. In contrast, when fragments of these elements were placed in front of the minimal CaMV 35S promoter in tandem (4×), strong GUS activity was measured and auxin-inducibility almost completely abolished (Ulmasov et al., 1995). Therefore, it is possible that alterations in the original structure of an auxin-inducible promoter region can result in strong constitutive activity. These findings are consistent with the hypothesis that the *VR-ACS1* promoter has become at least partially de-regulated in tobacco and *Arabidopsis*.

Our results clearly show that the *VR-ACS1* promoter can be used to drive strong constitutive expression of genes in tobacco and *Arabidopsis*. Although characterization studies have shown very consistent results in these two plant species, using transient and stable gene expression systems, a number of questions remain. How will the promoter behave in other untested species? Will it show constitutive expression in monocots? Will other cycloheximide-inducible promoters from mung bean or other species display a similar behavior when tested in heterologous hosts? Ultimately, characterization of the *VR-ACS1* promoter in stably transformed mung bean plants will shed light upon its regulatory mechanisms in its original system and lead to the identification of the *trans*-factors that control its expression.

The *VR-ACS1* promoter could prove a very valuable tool in biotechnology, and its value even further increased by additional research to determine its mechanism of action. We have already grown five generations of plants without incidence of gene silencing effects, another valuable characteristic sought in crop biotechnology. Unlike some of the other available constitutive promoters derived from plant viruses, the *VR-ACS1* promoter is of plant origin, which may prove to be a positive factor in public acceptance of GM technologies. Finally, artificial rearrangements of the *VR-ACS1* promoter such as tandem repeats of the core and/or 5'UTR region could result in further increases to expression levels as has been the case with other promoters. Further research is needed

to uncover the complex nature of *VR-ACS1* regulation in mung bean.

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